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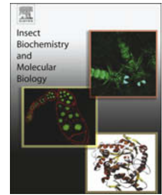

Short Communication

Purification of an active, oligomeric chitin synthase complex from the midgut of the tobacco hornworm

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ABSTRACT

Chitin formation depends on the activity of a family II glycosyltransferase known as chitin synthase, whose biochemical and structural properties are largely unknown. Previously, we have demonstrated that the chitin portion of the peritrophic matrix in the midgut of the tobacco hornworm, *Manduca sexta*, is produced by chitin synthase 2 (CHS-2), one of two isoenzymes encoded by the *Chs-1* and *Chs-2* genes (also named *Chs-A* and *Chs-B*), and that CHS-2 is located at the apical tips of the brush border microvilli. Here we report the purification of the chitin synthase from the *Manduca* midgut as monitored by its activity and immuno-reactivity with antibodies to the chitin synthase. After gel permeation chromatography, the final step of the developed purification protocol, the active enzyme eluted in a fraction corresponding to a molecular mass between 440 and 670 kDa. Native PAGE revealed a single, immuno-reactive band of about 520 kDa, thrice the molecular mass of the chitin synthase monomer. SDS-PAGE and immunoblotting indicated finally that an active, oligomeric complex of the chitin synthase was purified. In summary, the chitin synthase from the midgut of *Manduca* may prove to be a good model for investigating the enzymes' mode of action.

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1. Introduction

Chitin synthesis is based on a conserved biosynthetic machinery of which the chitin synthase (CHS) is the key enzyme, because it catalyzes the polymerization of *N*-acetylglucosamine by transferring the sugar portion of UDP-*N*-acetylglucosamine to the non-reducing end of the growing polymer (Merz et al., 1999). Chitin synthases are transmembrane family II glycosyltransferases, which consist of a central catalytic domain flanked by two transmembrane domains (Merzendorfer and Zimoch, 2003). Although chitin is a secretion product and deposited at the extracellular surface, the catalytic domain faces the cytoplasm. Consequently, chitin has to be translocated across the membrane, and this process is believed to be a function of the chitin synthase as well (Cohen, 2001). Although chitin synthases have been investigated extensively in fungal systems, basic properties of this class of glycosyltransferases are still poorly understood, mainly because sufficient purification procedures and heterologous expression systems are not available.

The lack of knowledge about chitin synthases is particularly evident in insect systems. As far as investigated, insects have only two genes encoding chitin synthases, *Chs-1* and *Chs-2* (also named *Chs-A and Chs-B*; Merzendorfer, 2006). As reported for *Manduca sexta* and *Tribolium castaneum*, *Chs-1* is specialized for chitin synthesis in the epidermal and tracheal cuticle, while *Chs-2* is specialized for chitin synthesis in the course of peritrophic matrix formation by midgut epithelial cells (Arakane et al., 2005; Hogenkamp et al., 2005; Lehane, 1997; Zimoch et al., 2005). While most studies that have analyzed chitin synthesis in insects focused on epidermal and tracheal cuticles, we have focused on the midgut chitin synthase. In the lepidopteran midgut chitin is secreted by columnar cells along with other components of the peritrophic matrix (Arakane et al., 2005; Hopkins and Harper, 2001), which protects the epithelium from mechanical damage and infections by pathogens (Lehane, 1997; Wang and Granados, 2001). Immunocytochemistry revealed that the chitin synthase from feeding larvae is localized at the very apical tips of the brush border microvilli formed by midgut columnar cells (Arakane et al., 2005; Zimoch and Merzendorfer, 2002). Moreover, *Chs* gene expression is regulated differentially during larval development. While *Chs-1* expression is up-regulated during molt and down-regulated during intermolt, *Chs-2* expression is down-regulated during molt and up-regulated during the intermolt (Zimoch et al., 2005). Next to the transcriptional regulation of *Chs* expression, posttranslational proteolytic

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activation appears to be involved in regulating enzyme activity. The zymogenic nature of chitin synthases was deduced from the observation that trypsin treatment stimulates chitin synthesis in various systems (Duran and Cabib, 1978; Merz et al., 1999; Merzendorfer, 2006; Roncero, 2002). Supportive evidence for the zymogenic character of chitin synthase was recently reported for CHS-2 from yeast, which is hyper-activated by a soluble but still unidentified yeast proteinase (Martínez-Rucobo et al., 2009). In the case of *Manduca*, we could show that trypsin stimulates chitin synthesis in crude midgut extracts, but not in membrane fractions, indicating that trypsin does not cleave the chitin synthase zymogen (Zimoch et al., 2005). However, we could identify a chymotrypsin-like endoproteinase CTLP1, which binds to the extracellular carboxyterminal domain of CHS-2 and might be involved in regulation of chitin synthesis (Broehan et al., 2007). As chymotrypsins are proteolytically activated by midgut trypsins whose secretion is induced by a feeding stimulus, chitin synthase activity and hence peritrophic matrix formation may be directly controlled by the nutrition state of the larvae.

In fungi, different attempts have been made to purify the active chitin synthase. However, only lower molecular weight fragments were reported to be associated with chitin synthesis when investigated by SDS-PAGE (Braun and Calderone, 1979; Duran and Cabib, 1978; Kang et al., 1984; Lending et al., 1991; Machida and Saito, 1993; Montgomery et al., 1984; Ruiz-Herrera et al., 1980; Uchida et al., 1996). Partial purifications with significant chitin synthase activity in microsomal fractions have been reported additionally from three insect sources (Cohen and Casida, 1980; Mayer et al., 1980; Ward et al., 1991). Here we report the purification of a chitin synthase from the midgut of *M. sexta*. Isolation of the chitin synthase from the *Manduca* midgut yielded a high molecular mass complex of 520 kDa suggesting that an active, oligomeric chitin synthase complex has been purified.

2. Materials and methods

2.1. Insects

Larvae of *M. sexta* (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h of light) at 27 °C using a synthetic diet modified according to Bell and Joachim (1974).

2.2. Measurement of chitin synthesis

Chitin synthesis was measured as described previously (Zimoch et al., 2005). In brief, the standard assay was performed in a volume of 100 µl and the reaction was initiated by the addition of 30 nCi UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine (specific activity: 285 mCi/mmol, GE Amersham). Incubation was carried out for different time periods in 1.5-ml Eppendorf polypropylene tubes at 30 °C. The reaction was stopped by adding 1 ml 1.5 M KOH followed by an incubation for 2 h at 100 °C. After filtration through 2.5 cm glass fibre filters (PALL; type A/E; 0.3 µm) and washing with 98% ethanol, radioactivity was measured as counts per minutes (cpm) using a Beckman liquid scintillation spectrometer. Each experiment was repeated at least three times with each single value determined in triplicates.

2.3. Purification of the midgut chitin synthase

Total midguts of twenty *Manduca* fifth instar larvae were isolated on a cooling plate. The Malpighian tubules were detached, the midgut was dissected and the gut content was removed together with the peritrophic matrix. Larval tissues were washed three times in CHS buffer (50 mM MOPS, 10 mM $MgCl_2$, 30 mM KCl, pH 6.5) and subsequently homogenized for 1 min with an Ultraturrax homogenizer (T25, Janke & Kunkel) at 25,000 rpm in 4 ml CHS buffer. The homogenates were differentially centrifuged in fixed-angle rotors at 4 °C for 10 min at 600 × *g*, 30 min at 10,000 × *g* and 1 h at 100,000 × *g*.

To solubilize the chitin synthase, the membrane vesicles of the 10,000 pellets were diluted with CHS buffer (f. c. 5 mg protein/ml) and treated with 0.5% (v/v) Triton-X-100 for 20 min at 30 °C in a rotatory incubator. After centrifugation for 1 h at 100,000 × *g* and 4 °C the supernatant was separated from the pellet, which was resuspended in 1 ml of CHS buffer. To remove any remaining particles and fat, the supernatant was filtrated through silanized fiberglass.

Each 2 ml of solubilized proteins were layered onto a discontinuous sucrose gradient (3 ml of 40%, 2 ml of 30%, 2 ml of 20% and 1.6 ml of 10% (w/v) sucrose in CHS buffer with 0.5% (v/v) Triton-X-100) and centrifuged in a vertical tube rotor for 90 min at 363,000 × $g\,(r_{av})$ and 4 °C. The gradient was then fractionated using a flexible-tube pump. The first 3 ml of the 40% fraction were discarded. The subsequent 30% and 20% fractions were each collected as 1 ml aliquots, yielding a lower and an upper fraction for both sucrose densities. The 10% sucrose fraction was collected as a lower 1 ml fraction and an upper 600 µl fraction. The proteins of the 10% fraction were subjected to anion exchange chromatography (MonoQ 5/5 HR, GE Amersham) at a flow rate of 0.5 ml/min. Binding was performed in a buffer of 20 mM Tris–HCl, 50 mM NaCl and 0.025% (v/v) Triton-X-100 (pH 8.1). Elution was carried out with a two-step gradient. The first step was at 0.2 M NaCl to remove weakly bound proteins. The second step was a linear gradient from 0.2 to 1 M NaCl (20 mM/min). Chitin synthase activity containing fractions were pooled and concentrated using a centrifugal filter device (Amicon Ultra 15, cut-off 10 kDa). Each 200 µl was loaded onto a Superdex 200 HR 10/300 GL gel chromatography column (GE Amersham). FPLC was performed with a flow rate of 0.5 ml/min using CHS buffer containing 0.025% (v/v) Triton-X-100. To prevent proteolytic degradation the preparation was carried out in the permanent presence of 5 mM Pefabloc SC (Serva).

2.4. Immunopurification

Membrane vesicles containing chitin synthases were enriched by immunoadsorption. For this purpose anti-CHS antibodies were coupled to an Affi-Gel Hz agarose support following the manufacturers' recommendations (BioRad). 10,000 × *g* membranes of the midgut tissue were resuspended in PBS buffer (pH 7.4) and bound to the affinity matrix. After washing the matrix each three times with 10 ml PBS buffer containing 500 mM or 50 mM NaCl, respectively, elution was carried out with 0.1 M glycine (pH 5–2.5). The eluates were immediately neutralized by addition of Tris–Base.

2.5. Gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970). Native PAGE was performed in discontinuous polyacrylamide gels (Ready Gel 10–20% Tris–HCl, pH 8.3) following the manufacturers' recommendations (BioRad). Proteins were either stained with Coomassie Blue (Schweikl et al., 1989) or silver (GE Amersham, silver staining kit). Calcofluor White staining was performed using a 0.01% (w/v) Fluostain I solution in CHS buffer (Sigma). After destaining the gel 3 times for 1 h in CHS buffer at room temperature, fluorescence was viewed with the VersaDoc Imaging system (BioRad).

2.6. Other methods

Protein concentrations were determined by the Amido Black method according to Wieczorek et al. (1990). Polyclonal antibodies

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